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I hereby certify that annexed is a true copy of the Provisional Specification as filed on 18 July 2003 with an application for Letters Patent number 527075 made by BLIS TECHNOLOGIES LIMITED.

Dated 5 August 2004.

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PROVISIONAL SPECIFICATION

TREATMENT OF MALODOUR

We, **BLIS TECHNOLOGIES LIMITED**, a New Zealand company of Level 10, Otago House, 481 Moray Place, Dunedin, New Zealand, do hereby declare this invention to be described in the following statement:

TREATMENT OF MALODOUR

FIELD OF THE INVENTION

This invention relates to methods of treating halitosis, and to the use of *Streptococcus salivarius* strains and compositions containing same in the prevention or treatment of halitosis.

BACKGROUND

Halitosis or bad breath is a common complaint characterised by the production of volatile sulfur compounds. The production of such compounds is generally associated with oral bacteria, particularly certain anaerobic species. These bacteria generally inhabit oral surfaces, and particularly periodontal pockets and the dorsa of the tongue surface.

The primary source of volatile sulphur compounds (VSC's) from the subgingival microflora is from microorganisms that can be both commensal and pathogenic. Previous culture-based studies have indicated that *Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, Micromonas micros* (formerly, *Peptostreptococcus*), *Bacteroides* species, *Campylobacter rectus, Eikenella corrodens, Desulfovibrio* species, *Treponema denticola, and Eubacterium* species amongst others are responsible for the production of VSC's that contribute to halitosis (as summarized by Loesche WJ, Kazor C. Periodontol 2000. 2002;28:256-79. and Khaira N, Palmer RM, Wilson RF, Scott DA, Wade WG. Oral Dis. 2000 Nov;6 (6):371-5.). However, recent non-culture based studies have shown that there are certain species associated with subjects that are either healthy or afflicted with halitosis. *Atopobium pavulum, Eubacterium sulci, Fusobacterium periodonticum, Dialister,* a phylotype of streptococci, a phylotype of the uncultivated phylum TM7, and *Solobacterium moorei* appeared to be present in subjects with halitosis. By contrast, *Streptococcus salivarius, Rothia mucilaginosa (Stomatococcus mucilaginosus),* and an uncharacterized *Eubacterium* (strain FTB41) were commonly detected only amongst healthy individuals (Kazor, C.E. et al., J. Clin Microbiol, Feb 2003, pp 558-563).

Over the years various methods have been developed and tried with varying success, to prevent or at least alleviate the problem of halitosis. Current treatments focus on anti bacterial regimes to reduce numbers of oral bacteria, or agents to mask or neutralise the

offensive odour. Oral rinses with chlorine dioxide have been shown to have some effect in the control of halitosis, but the levels of chlorine dioxide are in excess of accepted levels in drinking water, and are not recommended for ingestion. Current methods require complex physical, chemical or expensive regimes to be carried out and are typically only of short term effect, as the malodour-causing oral bacteria recover to former levels after treatment is stopped.

What is sought to treat halitosis is the replacement of the disease-causing organisms, with a non-virulent commensal microorganism. To serve as an effector strain in replacement therapy, the microorganism must be able to compete successfully with the pathogenic microorganism either via competitive action (eg for attachment sites), and/or antibiotic action, or inhibition by other metabolism-associated by-products.

In WO 01/27143 *S. salivarius* strains are identified which have utility in the treatment of dental caries caused at least in part by *S. sobrinus*. No activity was recorded against any anaerobic microorganisms. Moreover, the treatment of halitosis is nowhere contemplated in that document.

The present invention is broadly directed to methods of at least inhibiting growth of anaerobic microorganisms using BLIS-producing *S. salivarius* strains or compositions comprising same, or at least provides the public with a useful choice.

SUMMARY OF THE INVENTION

Accordingly, in one aspect the invention provides a method for at least inhibiting the growth of anaerobic bacteria sensitive to BLIS-producing *S. salivarius,* the method comprising contacting the sensitive bacteria with an inhibitory effective amount of a BLIS-producing *S. salivarius*, or an extract, or a composition or formulation containing same.

In a further aspect, the invention provides a method of prophylactic or therapeutic treatment of halitosis in an individual in need thereof, the method comprising administering to said individual a BLIS-producing *S. salivarius* extract, composition or formulation in an amount effective to at least inhibit growth of anaerobic bacteria or in an amount to allow effective colonisation in the oral cavity of the individual.

Preferably the *S. salivarius* are *Salivaricin B* producers.

Preferably, the anaerobic bacteria are *Eubacterium* and/or *Micromonas*, especially *Eubacterium saburreum* and *Micromonas micros*.

In a further aspect, the invention provides a method of controlling the incidence and severity of halitosis comprising introducing into the oral cavity of an individual susceptible to halitosis, a halitosis controlling amount of a BLIS-producing *S. salivarius*, extract, composition or formulation.

In one embodiment the halitosis is caused by *Eubacterium* or *Micromonas*, particularly *Eubacterium saburreum* and *Micromonas micros*..

Preferably, *S. salivarius* is administered as part of a lozenge, spray or other drug delivery device, confectionary, food, drink or nutraceutical.

The methods of the invention preferably include the preliminary step of pre-treating the individual to at least reduce the oral microflora already present.

The invention also relates to the use of BLIS-producing *S. salivarius*, extracts, compositions or formulations in the methods discussed above. Particlarly, to the use of the *S. salivarius* in the preparation of medicaments for use in treating halitosis.

In another aspect, the invention also relates to the use of BLIS-producing *S. salivarius* strains and active extracts in the methods discussed above for inhibiting, controlling, preventing or treating halitosis caused at least in part by *Eubacterium saburreum* and *Micromonas micros*.

Although the invention is broadly as described above, it will be appreciated by those persons skilled in the art that the invention is not limited thereto but also includes embodiments of which the following description gives examples.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is directed in a first aspect to a method for at least inhibiting the growth of anaerobic bacteria sensitive to BLIS-producing *S. salivarius*. The

method comprises contacting the sensitive bacteria with an inhibitory effective amount of a BLIS-producing *S. salivarius*, or an extract, or a composition or formulation containing same.

In another embodiment the invention relates to methods of prophylactially or therapeutically treating halitosis, and to methods of controlling the incidence and severity of halitosis as set out above.

Preferably, the *S. salivarius* strains for use in the invention are *Salivaricin B* producers with activity against anaerobic bacteria, particularly strains *Eubacterium saburreum* and/or *Micromonas micros*. BLIS-producing strains with activity against anaerobic bacteria include K12, and K30 both deposited with Deutche Sammlung von Mikroorganismen Und Zellkulturen GmbH, Mascheroder Weg 1 b, D-38124, Braunschweig, Germany on 8 October 1999, and 8 October 1999, and assigned Accession Nos. DSM 13084 and 13085 respectively.

Strain Sal 20P3 was deposited at the Australian Government Analytical Laboratories, 1 Suakin Street, Pymble, New South Wales, Australia in July 1992 under Accession No. AGAL 92/32401.

While *Salivaricin A* producer 20P3 has activity against *Micromonas*, *Salivaricin B* producers K12 and K30 have a broader range of activity against *Eubacterium* and *Micromonas* at least.

As noted above *Eubacterium* and *Micromonas* are considered causative agents in halitosis. While these BLIS-producing strains of *S. salivarius* are known to be active against gram-positive aerobic bacteria, their activity against anaerobic bacteria, and *Eubacterium* and *Micromonas* in particular, is unexpected. All the more so because BLIS-producing organisms are typically known to act against more closely related species.

These BLIS-producing *S. salivarius* are therefore useful as anaerobic antibacterial agents *per se* as well as therapeutically. In this context, "therapeutic" includes prophylactic treatment. Therapeutic uses include the treatment or prevention of anaerobic microbial infections, especially *Eubacterium* and *Micromonas* infections. The *S. salivarius* are particularly suitable for use against *Eubacterium saburreum* and *Micromonas micros*. Conditions amenable to treatment with the *salivarius* include halitosis and bad breath.

Extracts obtainable from the BLIS-producing *salivarius* strains are also useful in the invention. These active extracts may similarly be used in therapeutic formulations and methods. Extracts can be obtained using known art protocols, conveniently by cell culture and centrifugation.

A "therapeutic formulation" is a formulation appropriate for administration of an *S. salivarius* strain or extract herein, to an individual in need of same, particularly a halitosis-susceptible individual. In general, therapeutic formulations are composed of an *S. salivarius* strain or extract discussed above and an acceptable carrier, diluent and/or excipient.

An "acceptable carrier, diluent and/or excipient" means a vehicle for delivery of a *S. salivarius* strain or extract, to the individual, in which the vehicle is compatible with bacterial cell viability, or activity of the extract. Acceptable carriers suitable for use in the administration of viable *S. salivarius* strains and extracts are well known to those skilled in the art. Suitable carriers are generally inert and can be either solid or liquid.

In one embodiment, the carrier is a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers suitable for use with the *S. salivarius* strains herein include, but are not limited to, water, buffered saline solutions (e.g., phosphate-buffered saline), pharmaceutically acceptable culture media (e.g. BACa, TSBCaYE agar), or other solutions which maintain the viability of the bacterium. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. A variety of pharmaceutically acceptable carriers suitable for oral administration of viable or lyophilized bacteria are well known in the art (see, for example, *Remington's Pharmaceutical Sciences*, 18th ed., Gennaro, ed., 1990, Mack Publishing Co., Easton, Pa., incorporated herein by reference; and the pharmaceutical composition LACTINEX™, a commercially available formulation for oral administration of viable lactobacilli). Suitable solid carriers known in the art include, for example, magnesium carbonate; magnesium stearate; celluloses; talc; sugars such as fructose, sucrose, mannitol, lactose; starches; flours; oligosaccharides and skim milk, and similar edible powders, but are not limited thereto. Carriers for administration of extracts are similarly well known.

Typical diluents, by way of example, are: starches; lactose; mannitol; kaolin; calcium phosphate or sulphate; inorganic salts such as sodium chloride; and powdered sugars or celluloses.

The compositions may also include excipients such as tableting aids; resins; fillers; binders; lubricants; solvents; glidants; disintegrants; preservatives; buffers; flavourings; colourings; sweeteners; and fragrances as appropriate. A preferred excipient for tablet flowability and compactability is ProSolv™ (Penwest, NY, USA). A preferred sweetener is isomalt.

Typical binders include starch; gelatin; sugars such as lactose, fructose, and glucose; and the like. Natural and synthetic gums are also convenient, including acacia; alginates; locust bean gum; methylcellulose; polyvinylpyrrolidine tragacanth; Xanthan gum: and the like. Polyethylene glycol; ethyl cellulose; and waxes can also serve as binders. A currently preferred binder is Emdex™ (Penwest, NY, USA).

Lubricants to prevent sticking to the die during formation include slippery solids such as talc, silica, magnesium and calcium stearate, polyethylene glycol, stearic acid and hydrogenated vegetable oils.

Disintegrators are substances which swell when wetted to break up the lozenge and release the *S. salivarius* or extract. The disintegrators include starches; clays; celluloses; algins and gums; more particularly corn and potato starches; methylcellulose; agar; bentonite; wood cellulose; cation exchange resins; alginic acid; guar gum; citrus pulp; carboxymethylcellulose; powdered sponge; and sodium lauryl sulfate.

The *S. salivarius* strains or extracts herein can be formulated in any of a variety of compositions suitable for oral administration. For example, the *S. salivarius* strains can be formulated for administration as a lyophil or cell paste prepared from a *S. salivarius* culture, or can be directly administered to the oral cavity. The strain or extract can also be administered in the form of a mouthwash, mouth rinse, toothpaste, mouthspray, gargle, capsule, lozenge, syrup, floss, chewing gum, or chewable tablet but the forms are not limited thereto.

Therapeutic formulations may include food, confectionary or drink. In one embodiment, the foodstuff or drink is a dairy product-based food or drink including by way of example, yoghurt, cheese, milk, milk power, milk biscuits, and flavoured milks. In the case of confectionary, the formulation can be a chewing gum such as described in WO 00/05972. One preferred formulation employs freeze dried *S. salivarius* strains herein, in milk powder

formulations in a manner similar to that previously reported for the preparation of Bifidus Milk Powder (Nagawa et al. (1988); J. Dairy Sci. 71:1777-1782).

One orally administrable formulation of *S. salivarius* is a blend of freeze dried *S. salivarius* strains with skim milk powder or the like which has been flavoured to enhance palatability.

Presently preferred orally administrable formulations of *S. salivarius*, or extracts herein are lozenges, chewable tablets, or capsules. Lozenges are particularly preferred. A suckable lozenge preferably comprises an *S. salivarius* strain or extract, isomalt and emdex. The lozenge may be prepared by direct compression, wet granulation, or dry granulation. The lozenges may be coated according to well known pharmaceutical practice.

The therapeutic formulation can additionally contain nutrients to maintain the viability of the bacterium in the formulation. As noted above, the formulation can also contain flavouring agents, colouring agents, fragrances, or other compounds which increase the palatability of the composition and/or enhance patient compliance without compromising the effectiveness of the formulation. Methods for preparation of formulations for oral administration are well known in the art (see, for example, Remington's Pharmaceutical Sciences, 18th ed., supra, incorporated herein by reference).

For general antimicrobial use, formulations may also be produced for other methods of administration including topically administrable formulations but not limited thereto.

The formulations and compositions may further comprise one or more secondary antibacterial agents. These secondary agents may, for example, be antibiotics, or other antibacterial agent or antibacterial producing microorganisms. Preferably, the secondary antibacterial agent is a BLIS or BLIS producing microorganism. The BLIS may be one or more of salivaricin A, A_1, A_2 and B.

Secondary agents useful in such a composition may be odour masking or neutralising agents such as peppermint, chlorine dioxide, zinc, baking soda or other agents with a similar purpose.

Other ingredients useful in such a composition are anticariogenic agents, for example Xylitol, fluoride, and calcium.

Further ingredients useful in such a composition are agents that selectively enhance growth of desirable bacteria over non desirable organisms. These agents may, for example, be oligosaccharides such as Nutriose® FB.

In the treatment of halitosis, *S. salivarius* strains or extracts can be administered to any individual susceptible to halitosis, usually an individual in which *Eubacterium* and/or *Micromonas* colonises the oral cavity such that the halitosis is caused at least in part by *Eubacterium* and *Micromonas*.

The term "individual" as used herein includes humans, horses, dogs, cats, pigs, sheep, cattle, goats but is not limited thereto. Preferably, the individual is a human. The *S. salivarius* strains can be administered to the individual at any age, e.g. childhood, adolescence, or adulthood.

S. salivarius herein can be orally administered in a variety of ways. For example, in the form of compositions or formulations discussed above, or as suspensions, sustained release formulas (e.g. an oral implant containing the *S. salivarius* strain) or lyophil powders. The *S. salivarius* strains can also be administered by direct application of a lyophil, culture, or cell paste to the oral cavity of the individual. Any mode of administration is suitable as long as the therapeutic formulation is applied to the oral cavity. In one embodiment, the *S. salivarius* or extracts are administered by applying directly to the tongue of the individual, e.g. by brushing.

In general, the amount of *S. salivarius* administered to the individual will be an amount effective for replacement of halitosis-causing anaerobic bacteria strains, or at least *Eubacterium* and/or *Micromonas* in the oral cavity of the host. "An amount effective for replacement of halitosis-causing anaerobic bacterial strains or at least *Eubacterium* and/or *Micromonas* in the oral cavity of the host" means an amount effective for oral cavity colonisation by the *S. salivarius* strain, and significant reduction of the resident halitosis-causing anaerobic bacteria (e.g. by competition between the bacteria for nutrients and/or by the production of BLIS by the *S. salivarius* strain).

The term "unit dose" when used in reference to a therapeutic formulation herein refers to physically discrete units suitable as unitary dosage for the individual, each unit containing a predetermined quantity of active material (viable *S. salivarius* or active extract thereof)

calculated to produce the desired therapeutic effect in association with the required diluent, carrier, or excipient.

Specific dosages can vary widely according to various individual variables including size, weight, age, disease severity (e.g. the tenacity and/or number of halitosis-causing resident bacteria) and responsiveness to therapy (e.g. the susceptibility of the individual's oral cavity to colonisation). Methods for determining the appropriate route of administration and dosage may be determined by the consumer as they deem appropriate, or on a case-by-case basis by an attending dentist or other clinician. Such determinations are routine to one of ordinary skill in the art (see for example, *Remington's Pharmaceutical Sciences*, 8th ed., Gennaro, ed., Mack Publishing Company, Easton, Pa., 1990).

In general, the number of *S. salivarius* administered to the individual will range from about 10^2 to 10^{15} bacteria, preferably from about 10^3 to 10^{14} bacteria, more preferably from about 10^5 to 10^{12} bacteria, normally about 10^9 to 10^{10} colony forming units (CFU) per dose. One formulation employs 3.8×10^9 CFU/lozenge.

Multiple doses of the *S. salivarius* strain can be administered to achieve oral cavity colonisation and replacement of the resident, halitosis-causing strains, particularly *Eubacterium* and/or *Micromonas* of the individual. The *S. salivarius* strain or extract may need to be administered to the patient once only or more usually repeatedly. Repeat treatments may be once a month, once a week, once a day, twice a day, or as may otherwise be required. Conveniently, the administration may be effected as part of the patient's routine dental care, e.g. as a component of a lozenge, gum, toothpaste, floss, or mouthwash.

To facilitate colonisation, in one embodiment the treatment method of the invention includes a preliminary step of pre-treating the individual to at least reduce the normal microflora present in the oral cavity, including halitosis causing organisms. This pre-treatment comprises the step of administering an antimicrobial agent such as chlorhexidine, triclosan, lactoperoxidase, green tea, or pineapple juice (freeze dried), but not limited thereto, may also include physical removal methods such as brushing or scaping, or may follow a prescribed course of antibiotics such as tetracyclines, penicillin, erythromycin, metronidazole, or amoxycillin administered to said individual. *S. salivarius*, extracts, formulations or compositions containing same are then administered to the depopulated environment to repopulate same.

A currently preferred treatment protocol for halitosis comprises pre-treatment by scraping the tongue brushing teeth and tongue with antibacterial toothpaste;; gargling with chlorhexidine; then taking a lozenge. A lozenge is administered 1-4 hours, preferably 2 hours after the pre-treatment. This is followed by administration of a further 2-5, preferably 3 lozenges through the day at intervals of 1-4 hours, preferably every 2 hours. This protocol is followed for 2-4 days to facilitate colonisation. For maintenance purposes 1, 2, or 3 lozenges, usually 1 to 2 lozenges are taken each day following ordinary tooth brushing. The regime is continued for as long as required.

Successful colonisation of the individual's oral cavity by the *S. salivarius* strain can be established by culturing the bacteria of the individual's oral cavity, and identifying the *S. salivarius* strain by, for example, BLIS production or other methods well known in the art for bacterial strain identification.

The methods and uses of the invention may further comprise the use of one or more secondary antibacterial agents as discussed above.

Where the term comprise, comprises, comprised or comprising are used in this specification, they are to be interpreted as specifying the presence of the stated features, integers, steps or components referred to, but not to preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

Various aspects of the invention will now be illustrated in a non-limiting way by reference to the following experimental section.

EXPERIMENTAL

Deferred Antagonism Test of Anti-bacterial Activity

The spectrum of inhibitory activity of *Streptococcus salivarius* K12 was established by use of a deferred antagonism test, essentially as described by Tagg and Bannister (J. Med. Microbiol. 1979;12:397). In brief, a 1-cm wide diametric streak culture of K12 (producer strain) was inoculated onto blood agar-calcium medium. Following incubation in a 5% CO_2 atmosphere, for 24 hours at 37°C, the macroscopic cell growth was removed with a glass slide and residual cells on the agar surface were killed by exposure to chloroform vapours for 30 minutes. The agar surface was then aired for 30 minutes. Indicator strains, which had been

grown for 48 hour on blood agar-calcium plates, were suspended in Todd Hewitt broth and used to inoculate at 90-degree angles across the line of the original streak culture with the use of sterile cotton swabs, under anaerobic conditions. After incubation for 48 hours in an anaerobic environment at 37°C the extent of inhibition of each indicator strain was recorded. The scoring system for the measurement of inhibition was the following: a negative sign (-) denoted no inhibition of the indicator microorganism; a positive symbol (+) indicated that there was some inhibition on the plate where the producer microorganism had grown, but not exclusively over that entire region; two positive symbols (++) denoted that the indicator strain was inhibited where the producer strain was grown; when three positive symbols (+++) were used, growth of the indicator strain was at least 5 mm away from where the producer strain had grown; and four positive symbols (++++) represented inhibition of the indicator strains beyond 5 mm of growth.

Table 1. BLIS Sensitivities of Microoganisms Implicated in Halitosis and Control

			Bacteriocin gene		Control strain	Inhibition of growth of organisms implicated in halitosis	
Strain	Properties	P-type[1]	*sal* A	*sal* B	*S. anginosis* (Indicator 3)	*Eubacterium saburreum* ATCC 33271	*Micromonas micros* ATCC 33270
K12	SAL_A & B producer	777	+	+	++++	++++	++++
NR	SAL_B producer only	777	-	+	++++	++	++++
20P3	SAL_A producer only	677	+	-	-	++	None
MU Neg.	Non BLIS producer	000	-	-	-	None	None

[1]On blood agar

INDUSTRIAL APPLICATION

BLIS-producing *S. salivarius* strains, particularly *Salivaricin B* producing strains against a number of microorganisms implicated in halitosis (Table 1). The strains and related active extracts herein therefore have application in methods of therapeutically treating individuals against the harmful effects of *Eubacterium* and *Micromonas* infection, especially in the oral cavity. These methods include treatment of halitosis in which these organisms are the primary causative agents. The *S. salivarius* extracts and compositions of the invention also have application in the treatment of sore throats.

It will be appreciated that the above description is provided by way of example only and that variations in both the materials and techniques used which are known to those persons skilled in the art are contemplated.

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